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Enhanced D-ribose production by genetic modification and medium optimization in *Bacillus subtilis* 168

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Abstract−D-ribose, a five-carbon sugar with diverse applications, is mainly produced by transketolase (*tkt*)-deficient *Bacillus subtilis* (*B. Subtilis*). We constructed *B.subtilis* SFR-3A by replacing the corresponding sites of *B. subtilis* 168 with the mutation site of *tkt* in the "wild" D-ribose high-producing strain *B. subtilis* SFR-4, resulting in 5.29 g/L of D-ribose. In the meantime, *B.subtilis* SFR-159 was constructed by completely removing the *tkt* gene of *B. subtilis* 168 with a higher production of 6.21 g/L. Through medium optimization, batch fermentation of SFR-3A and SFR-159 gave the best result of 27.56 g/L and 29.89 g/L, which corresponds to productivity of 0.51 g/L/h and 0.41 g/L/h, respectively. In this work, SFR-3A showed more latent capacity over SFR-159 as to productivity and had greater potential to serve as a platform for D-ribose production.

Keywords: *Bacillus subtilis*, D-ribose, Transketolase, Genetic Modification, Fermentation

INTRODUCTION

D-ribose is a five-carbon sugar which plays an exceptionally important role in the metabolism of plant and animal cells by being a major constituent component in ribonucleic acid, ATP, NADH, NADPH and FAD [1]. Its application areas are mainly in the commercial synthesis of riboflavin as well as flavor enhancers [2]. In recent years, there has been increased interest in its role in clinical drugs to improve cardiac function [3], and pharmaceutical intermediates for the synthesis of antiviral and antitumor drugs [4].

Compared with chemosynthesis, D-ribose production by fermentation has many advantages such as mild process, lower production costs, wide raw materials sources, efficient purification and less environmental pollution. The biological synthesis of D-ribose occurs in the pentose phosphate pathway (PPP). D-xylose-5-P and together with D-Ribose-5-P, are converted by transketolase (*tkt*) to D-glyceraldehyde-3-P and D-heptanone-7-P. If transketolase is deactivated, D-ribose-5-P may accumulate inside the cells and hence is dephospholyated to D-ribose by nonspecific phosphatases [5]. The deficiency of tkt could lead to D-ribose accumulation in a variety of microorganisms, including *Saccharomyces cerevisiae* [6], *Escherichia coli* [7], *Bacillus pumilus* [8-10] and *Bacillus subtilis* [11]. However, only production of D-ribose by transketolase-deficient *Bacillus* strains spp. has been proven to be commercially viable [1]. The vast majority of D-ribose high-producing *B. subtilis* strains are the "wild" type which are difficult for transformation [12]. To date, most studies seeking to improve D-ribose production have focused on mutation breeding [1], culture medium optimization [13-15], metabolic regulation [16-18]. However, there have been only few reports about the research on the construction of engineering strain [19]. Fortunately, as a well-characterized model strain, *B. subtilis* 168 is an attractive platform for bioproduction due to its clear genetic background [20], ease of genetic manipulation and inherent safety. Through genetic modification of *tkt* in *B. subtilis* 168, metabolic flux might redirect towards D-ribose synthesis pathway.

As previously shown, a transketolase-deficient *B. subtilis* strain SFR-4 was isolated by mutagenesis which was able to produce 56 g/L D-ribose [21]. However, the negative mutation by multiple random mutagenesis decreased the viability of the strain, thus hampering its application in industrial production. By comparing the D-ribose biosynthesis genes of SFR-4 and *B. subtilis* subsp. Natto BEST195 which has close relationship with SFR-4, it was found that the major differences were detected in *tkt* gene and all mutational sites presented at 1,950 to 2,004 bp. In this paper, we constructed a strain SFR-3A by replacing the corresponding sites of *B. subtilis* 168 with the 54 bp mutation regions of the *tkt* gene of SFR-4, resulting in an titer of 5.29 g/L D-ribose. By fermentation optimization, 27.56 g/L of D-ribose could be formed. Moreover, compared with the strain SFR-159 which has complete knockout of *tkt*, SFR-3A had advantages in improving the productivity of D-ribose and showed more latent capacity from the point of industrial production.

MATERIALS AND METHODS

1. Strains, Plasmids, Primers and Media for Routine Cultivations

All strains, plasmids and primers used in this study are listed in Table 1 and Table 2. *B. subtilis* 168 was utilized for homologous recombination and was cultivated in Luria-Bertani (LB) or the appropriate selection medium. LB medium consisted of 10 g/L peptone, 5 g/L yeast powder and 10 g/L NaCl, and was supplemented with

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Table 1. Strains and plasmids used in this study

B. subtilis strains, or plasmids	Characteristic(s)	Source or references
Strains		
SFR-4	The wild type D-ribose high-producing strain isolated by mutagenesis	Lab stock
168	*trpC2*	Lab stock
SFR-3A zeo^r	*B. subtilis* 16.8 replacing the corresponding sites with the mutation site of *tkt* in SFR-4, harboring lox72-zeo-lox66 cassette.	This work
SFR-159 zeo^r	*B. subtilis* 168 *Δ*tkt::(lox72-zeo-lox66 cassette)	This work
SFR-3A	*B. subtilis* 168 replacing the corresponding sites with the mutation site of *tkt* in SFR-4	This work
SFR-159	*B. subtilis* 168 *Δtkt*	This work
Plasmids		
p7Z6	pMD18-T containing *lox71-zeo-lox66* cassette	[23]
pDGC	pDG148 containing cre fused to Pspac promoter	[23]

Table 2. Oligonucleotides used in this study

Primer name	Sequence 5′-3′
TKT1-F	AGCAGCATGGAAGCTTGCAG
TKT1-R	TGTATGCTATACGAACGGTATTATCTTGATACTATATAGAAACATCTCAAGGCG
TKTUP-F	CAGGAATACATAGAGAATGAAGAGACTG
TKTUP-R	TGTATGCTATACGAACGGTAAAAAATCCCCTTCCTTATGTGTTGTAC
TKTDN-F	CATACATTATACGAACGGTAGCTTTTGAAAGAGGATGAGTCAAATC
TKTDN-R	CACTTCAAAGAATATCGTTTCTGCC
zeo-F	TACCGTTCGTATAGCATACATTATACG
zeo-R	TACCGTTCGTATAATGTATGCTATACGAAG

25 μg/mL zeocin and 50 μg/mL kanamycin when necessary.

2. PCR-based Fusion of Antibiotic Resistance Marker Cassette with Long-flanking Homology (LFH) Regions

Fusion of the antibiotic resistance marker cassette with LFH regions by PCR was done as described by Shevchuk et al. [22]. In brief, it was carried out as follows.

(1) The *lox71-zeo-lox66* resistance marker cassette was amplified from vector p7Z6 [23] with primers *zeo*-F/*zeo*-R. With the genomic DNA of *B. Subtilis* SFR-4 as template, 1,500-2,004 bp of *tkt* was amplified with primers TKT1-F/TKT1-R. Then, applying the genomic DNA of *B. subtilis*168 as template, primers TKTDN-F/TKTDN-R were used to amplify 500 bp fragments flanking *tkt* gene sequence at its back end. Extensions of 20 nucleotides (nt) that were complementary to the 5′ and 3′ ends of the amplified *lox71-zeo-lox66* resistance marker cassette were added to the 5′ end of the reverse and forward primers of the front and back flanking regions, respectively. Then, the *lox71-zeo-lox66* cassette and these two region fragments were fused by PCR to construct the *tkt*-SFR-4 cassette.

(2) Similarly, applying the genomic DNA of *B. subtilis*168 as template, two 500-bp DNA fragments flanking the *tkt* gene sequence at its front and back ends were amplified with two primer pairs TKTUP-F/TKTUP-R, and TKTDN-F/TKTDN-R, respectively. Then, the *lox71-zeo-lox66* cassette and these two region fragments were fused by PCR to construct the *tkt*-DE-168 cassette.

In all cases, PCR was conducted with PrimeSTARR HS DNA Polymerase (Takara Biotechnology, Dalian, China), and all PCR products were gel purified with extraction from agarose using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (Takara Biotechnology, Dalian, China).

3. Strain Construction

The TKT-SFR-4 cassette and the TKT-DE-168 cassette were introduced into *B.subtilis* 168 by the Spizizen method [24]. And then the strains were inoculated onto LB plates containing 25 μg/mL zeocin, respectively. Selection plates were incubated at 37 °C for 24 h. The transformants were selected and confirmed by selecting monoclonal colony PCR analysis, yielding SFR-3A*zeo*^{r} and SFR-159*zeo*^{r}. The two strains were transformed with the plasmid pDGC, and incubating on LB agar containing 50 μg/mL kanamycin, respectively. The two transformants with pDGC were selected and confirmed by selecting monoclonal colony PCR analysis. Thereafter the selected transformants were transferred into LB both containing 1 mM IPTG and incubated for 8 h. Cells were diluted and spread on LB agar. 50 colonies of each strain were transferred into LB agar containing zeocin. The Zeo^s colonies were selected and confirmed by selecting monoclonal colony PCR analysis, yielding SFR-3A and SFR-159, respectively.

4. D-ribose Production and Medium Optimization

Cultivation for flask-scale D-ribose always entailed the following: *B. subtilis* strains were cultivated for 16 h at 37 °C with constant agitation in seed culture medium and then reinoculated to an initial OD_{600} of 0.2 in 50 mL medium and shaken at 180 rpm.

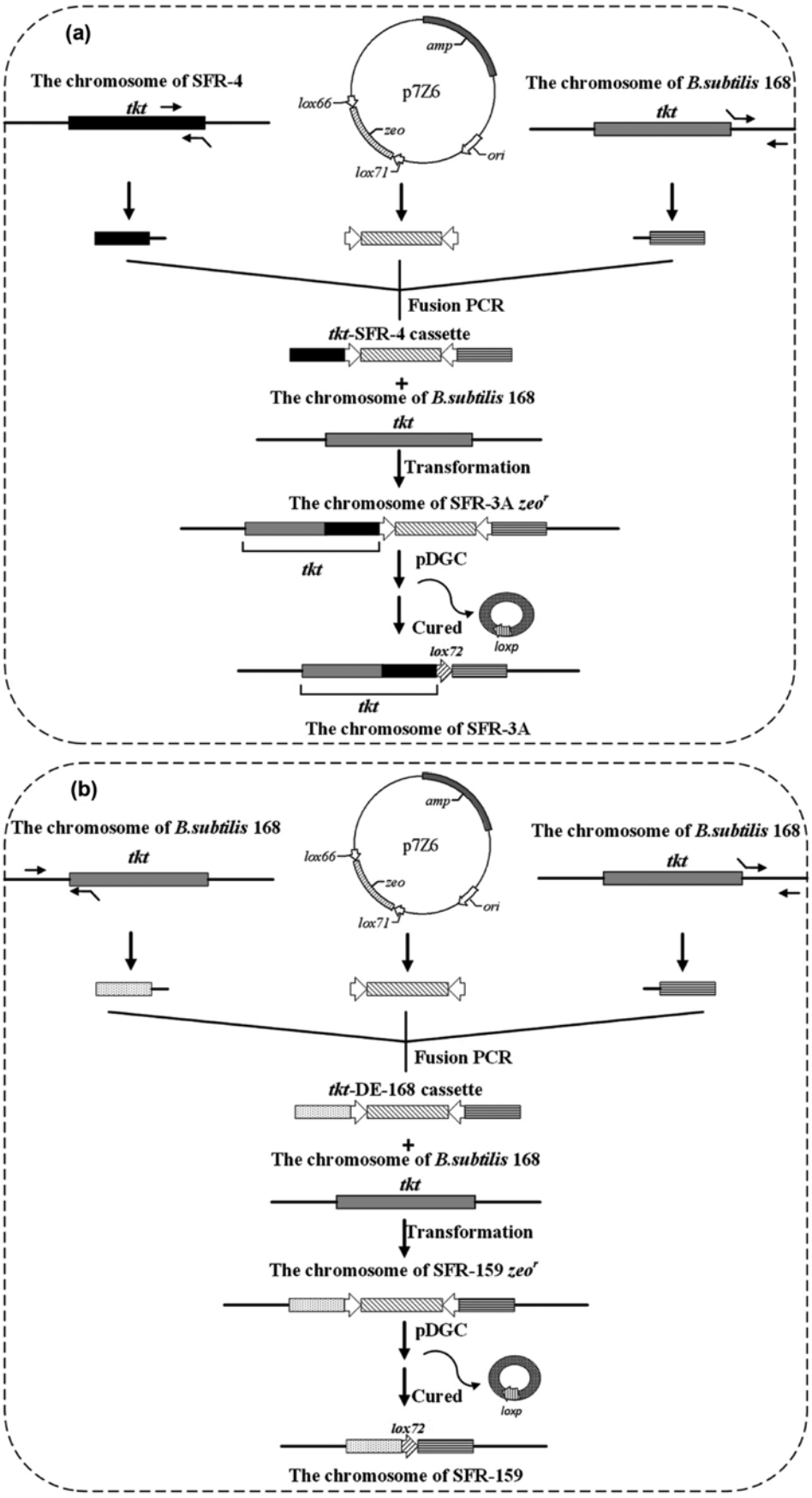

Fig. 1. Schematic drawing of the construction of *B. subtilis* SFR-3A (a) and SFR-159 (b).

The seed culture medium contained 20 g/L glucose·H_2O, 5 g/L yeast extract, 3 g/L corn steep dry and 3 g/L $(NH_4)_2SO_4$ at pH 6.5. The initial fermentation medium contained 120 g/L glucose·H_2O, 5 g/L yeast extract, 3 g/L corn steep dry and 3 g/L $(NH_4)_2SO_4$ at pH 6.5. Different concentrations of nitrogen source in initial fermentation medium were tested to investigate the effect of medium formulation on D-ribose production in SFR-3A. Fermentations were conducted for 96 h and then D-ribose production was detected.

5. Bioreactor Fermentations

Bioreactor fermentations were conducted as batch processes using a 5-L stirred fermenter (Shanghai Baoxing Bioengineering Equipment Co., Shanghai, China). The medium formulation contained 120 g/L glucose, 10 g/L yeast extract, 5 g/L corn steep dry and 5 g/L $(NH_4)_2SO_4$. Seed cultures were grown in 50 mL for 16 h and inoculated to the bioreactors with an initial broth volume of 3.5 L at an initial OD_{600} of 0.15. Oxygen was supplied using a constant air input flow rate of 0.5 v/(v*min), and the bioreactor pressure was maintained at 0.06 Mpa. The dissolved oxygen concentration was maintained at 15% of saturation by varying the impeller agitation speed. The pH of the culture was maintained at 6.5, and temperature was maintained at 37 °C.

6. Analytical Methods

The cell concentrations were determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer [25]. Glucose in the fermentation broth was measured by glucose-glutamate analyzer SBA-40D (Biology Institute of Shandong Academy of Science, Jinan, China). D-ribose was detected by high-performance liquid chromatography (Ultimate3000 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Hypersil NH_2 Column 4.6 mm×250 mm, Elite, CHN) under the following conditions: sample volume 10 L, mobile phase 80% (v/v) acetonitrile, flow rate 0.6 mL/min and column temperature 60 °C.

RESULTS AND DISCUSSION

1. Construction of Transketolase-deficiency Strain

The TKT-SFR-4 cassette and the TKT-DE-168 cassette were introduced into *B.subtilis* 168 to generate SFR-3A*zeo*^{r} and SFR-159*zeo*^{r}, respectively. Site-specific recombination systems such as Cre/lox had much higher recombination efficiency than the endogenous recombination systems, making them ideal for many genetic manipulation strategies. In this work, the plasmid pDGC was introduced into SFR-3A*zeo*^{r} and SFR-159*zeo*^{r}. Then, the *lox71-zeo-lox66* cassettes in two strains were evicted through transient Cre recombinase expression. We constructed no resistant strains by the *Cre/loxP* recombination system, referred to as SFR-3A and SFR-159, respectively (Fig. 1).

SFR-3A was constructed by replacing the corresponding sites of *B. subtilis* 168 with the mutation site of *tkt* of SFR-4. This genetic manipulation resulted in an initial D-ribose production of 5.29 g/L after 96 h shake-flask cultivation, while *B. subtilis* 168 could not produce D-ribose. By contrast, we constructed SFR-159 by completely removing *tkt* of *B. subtilis* 168, which produced 6.21 g/L D-ribose in the same condition.

2. Optimization of Culture Medium

Cultivation medium was optimized to further improve D-ribose levels. We first assessed the D-ribose synthesis by monitoring over the course of four days in SFR-3A (Fig. 2). In the initial fermentation medium, *B.subtilis* SFR-3A grew poorly and consumed the carbon source in a relatively slow speed, and consequently, merely a small quantity of D-ribose was formed. To improve the produc-

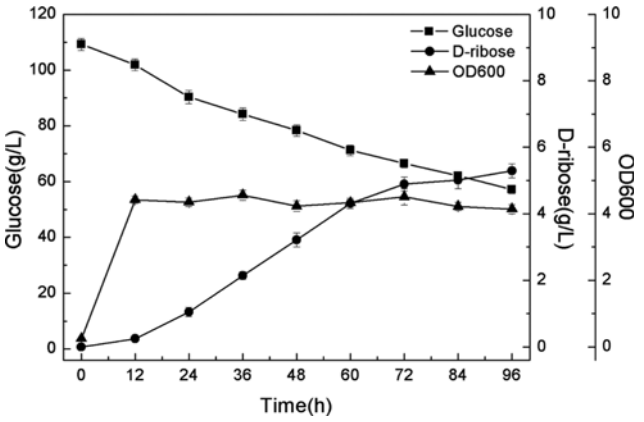

Fig. 2. Time profiles of shaking flask fermentation by *B. subtilis* SFR-3A. Cultivations were performed in a 500 mL shake-flask containing 50 mL the initial fermentation media at pH 6.5 and 37 °C.

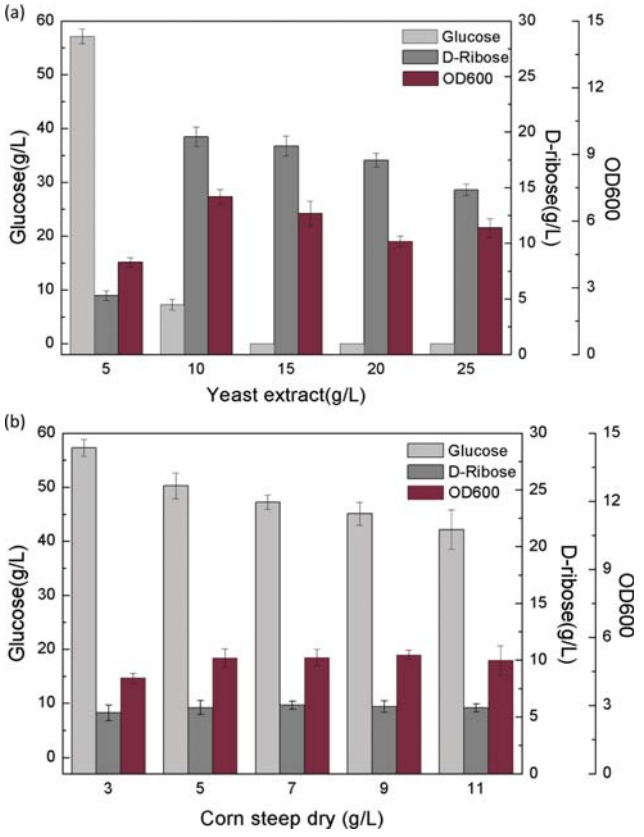

Fig. 3. Effect of initial yeast extract (a) and corn steep dry (b) concentrations on D-ribose fermentation by SFR-3A. Cultivations were performed in a 500 mL shake-flask containing 50 mL fermentation media at pH 6.5 and 37 °C for 96 h.

Table 3. Effect of different concentrations of yeast extract and corn steep dry addition on D-ribose fermentation by SFR-3A. Cultivations were performed in a 500 mL shake-flask containing 50 mL fermentation media at pH 6.5 and 37 °C for 96 h

No.	Yeast extract (g/L)	Corn steep dry (g/L)	OD_{600}	Glucose (g/L)	D-ribose (g/L)
1	7.5	3	5.03±0.85	25.8±1.1	12.13±0.59
2	7.5	5	7.18±0.42	22.9±0.9	14.15±0.47
3	7.5	7	7.86±0.46	18.4±0.5	16.01±0.58
4	10.0	3	7.09±0.22	7.25±0.8	19.61±0.45
5	10.0	5	7.46±0.72	3.21±0.7	22.39±0.86
6	10.0	7	7.61±0.67	0	21.53±0.58
7	12.5	3	7.58±0.56	0	21.14±0.67
8	12.5	5	7.62±0.86	0	19.56±0.74
9	12.5	7	7.49±0.64	0	19.52±1.04

tion of D-ribose, the effect of nitrogen sources on D-ribose fermentation was first investigated in shake flasks. According to the recent reports, the complex organic nitrogen source would be beneficial for the production of D-ribose [12,14,15]. Therefore, *B.subtilis* SFR-3A was cultivated in the initial fermentation medium supplemented with various concentrations of yeast extract and corn steep dry, respectively. The results indicated that the synthesis of D-ribose was significantly promoted by the increasing concentration of yeast extract (Fig. 3(a)). The SFR-3A exhibited a 270.13% improved D-ribose titer with 10 g/L yeast extract addition. Besides, it showed a 87.31% reduction in residual glucose and a 71.26% increase in biomass (OD_{600}). The fermentations with low concentrations of yeast extract addition (5 g/L) were less productive due to its low cell mass. However, higher amount (>15 g/L) of yeast extract addition decreased D-ribose production as the data show. Corn steep dry is a cost effective medium composition due to its high content of nitrogen source, water soluble vitamins, amino acid, minerals, and other growth factors [26]. Previous report also proved that corn steep dry as efficient nitrogen source nutrient and growth factor for industrial D-ribose fermentation [27]. But in our study, no significant positive effect of corn steep dry on D-ribose fermentation was observed because of low cell biomass (Fig. 3(b)). It was noted that corn steep dry was commonly referred to as the effective supplement of yeast extract for fermentation industry. And in this connection, we attempted to optimize D-ribose production by adjust the combinations of yeast extract and corn steep dry (Table 3). The maximum D-ribose production (22.39 g/L) was achieved under the optimum combination of 10 g/L yeast extract and 5 g/L corn steep dry. It increased 14.35% D-ribose yield compared to single nitrogen source. In general, ammonia has a strong effect on the product formation profile and fermentation physiology of an organism and has been shown to be used for large-scale D-ribose production [1]. To this end, the effect of $(NH_4)_2SO_4$ on D-ribose production was also optimized; 5 g/L $(NH_4)_2SO_4$ showed significant increase of D-ribose (26.25 g/L) (Fig. 4). For $(NH_4)_2SO_4$ concentrations of 3 and 5 g/L, the glucose consumption and cell biomass were not influenced by the concentration. While, it showed a 17.71% increase in D-ribose yield. It indicated that $(NH_4)_2SO_4$ could be beneficial for the transformation from glucose to D-ribose. Meanwhile, the culture medium of SFR-159 had been optimized, and it was consistent with SFR-3A.

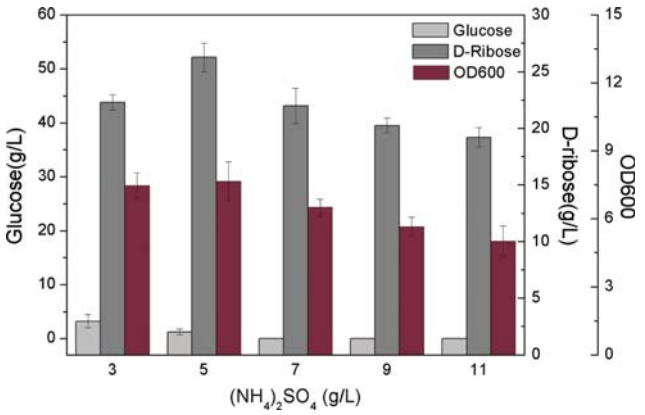

Fig. 4. Effect of $(NH_4)_2SO_4$ concentrations on D-ribose fermentation by SFR-3A. Cultivation was performed in a 500 mL shake-flask containing 50 mL the complex nitrogen source enhancing source medium with the different concentrations of $(NH_4)_2SO_4$ at pH 6.5 and 37 °C for 96 h. In addition, the media contained 120 g/L glucose, 10 g/L yeast extract, 5 g/L corn steep dry.

These results indicated that SFR-3A and SFR-159 needed more nitrogen source for self-growth and D-ribose biosynthesis compared to SFR-4.

3. Bioreactor Fermentation of SFR-3A and SFR-159

The D-ribose fermentation characteristics of SFR-3A and SFR-159 were compared by batch fermentations. Fig. 5(a) depicts the profiles of cell growth, glucose consumption and D-ribose production of SFR-3A. The maximum biomass (OD_{600}) of 10.25 was obtained at 12 h. Glucose was completely consumed at 54 h. D-ribose was accumulated after the mid-exponential phase and a maximum yield was 27.56 g/L. Batch fermentation of SFR-159 is shown in Fig. 5(b). A maximum biomass (OD_{600}) of 9.89 was obtained at 12 h. Complete utilization of glucose by SFR-159 was at 72 h, and a finial D-ribose concentration of 28.15 g/L was achieved. However, the D-ribose productivity of SFR-3A was 24.39% higher than SFR-159 up to 0.51 g/L/h. Transketolase is a metabolic enzyme involved in the non-oxidative pentose phosphate pathway connecting glycolysis and the PP pathway. Completely knocked out of *tkt* resulted in D-ribose accumulation [5], but cell growth and metabolism were inhibited at the same time, which limited the applica-

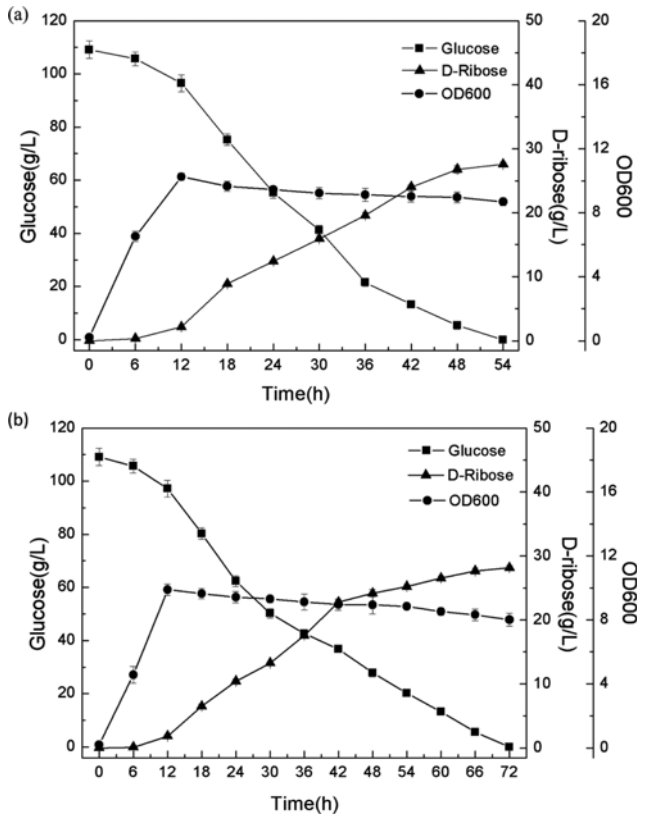

Fig. 5. Fed-batch cultivation of SFR-3A (a) and SFR-159 (b). The medium formulation contained 120 g/L glucose, 10 g/L yeast extract, 5 g/L corn steep dry and 5 g/L $(NH_4)_2SO_4$. Oxygen was supplied using a constant air input flow rate of 0.5 v/(v*min) and the bioreactor pressure maintained at 0.06 Mpa. The dissolved oxygen concentration was maintained at 15% of saturation by varying the impeller agitation speed between 200 rpm and 350 rpm. The culture's pH was maintained at 6.5 using 2.5 M NaOH and 4 M H_2SO_4, and temperature was maintained at 37 °C.

tion in industrial production. So it was assumed that the transketolase of SFR-3A might had part activity, which had less impact on cell growth and metabolism. So these results demonstrated that SFR-3A had more latent capacity to serve as a platform for industrial D-ribose production.

CONCLUSIONS

The study presents a metabolic engineering and optimization strategy for the production of D-ribose in *B. subtilis*. We constructed *B. subtilis* SFR-3A by replacing the corresponding sites of *B. subtilis* 168 with the mutation site of *tkt* of D-ribose high-yielding strain SFR-4. It increased D-ribose production to nearly 27.56 g/L with an overall productivity of 0.51 g/L/h under bioreactor fermentations. Compared with the strain SFR-159 complete knockout of *tkt,* SFR-3A showed great potential as a platform organism for metabolic engineering and a promising biomass material for D-ribose industrial production. In summary, this work provides a new view toward the development of alternative, industrially relevant hosts for D-ribose production.

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REFERENCES

1. K. I. Sasajima and M. Yoneda, *Agr. Biol. Chem.*, **35**(4), 509 (1971).
2. P. De Wulf and E. J. Vandamme, *Appl. Microbiol. Biotechnol.*, **48**, 141 (1997).
3. D. F. Pauly and C. J. Pepine, *J. Cardiovasc. Pharmacol. Ther.*, **5**(4), 249 (2000).
4. D. Maccarter, N. Vijay, M. Washam, L. Shecterle and H. Sierminski, *Int. J. Cardiol.*, **137**(1), 79 (2009).
5. Y. C. Park, S. G. Kim, K. Park, K. H. Lee and J. H. Seo, *Appl. Microbiol. Biotechnol.*, **66**, 297 (2004).
6. M. H. Toivari, H. Maacheimo, M. Penttilä and L. Ruohonen, *Appl. Microbiol. Biotechnol.*, **85**, 731 (2000).
7. H. C. Park, Y. J. Kim, C. W. Lee, Y. T. Rhlo, J. W. Kang, D. H. Lee, Y. J. Seong, Y. C. Park, D. Lee and S. G. Kim, *Process. Biochem.*, **52**, 73 (2017).
8. R. K. Srivastava, S. K. Maiti, D. Das, P. M. Bapat, K. Batta, M. Bhushan and P. P. Wangikar, *J. Ind. Microbiol. Biotechnol.*, **39**, 1227 (2012).
9. R. K. Srivastava and P. P. Wangikar, *J. Chem. Technol. Biotechnol.*, **83**, 1110 (2008).
10. R. K. Srivastava, R. Jaiswal, D. Panda and P. P. Wangikar, *Biotechnol. Bioeng.*, **102**, 1387 (2009).
11. P. D. Wulf, W. Soetaert, D. Schwengers and E. J. Vandamme, *J. Appl. Microbiol.*, **83**, 25 (1997).
12. R. Nijland, J. G. Burgess, J. Errington and J. W. Veening, *PLOS ONE*, **5**(3), e9724 (2010).
13. T. Fang, X. C. Chen, N. Li, H. Song, J. X. Bai, J. Xiong and H. J. Ying, *Korean J. Chem. Eng.*, **27**(6), 1725 (2010).
14. Y. C. Park and J. H. Seo, *J. Microbiol. Biotechnol.*, **14**(4), 665 (2004).
15. Z. Wei, J. Zhou, W. J. Sun, F. J. Cui, Q. H. Xu and C. F. Liu, *BioMed Research International*, Article ID 535097 (2015).
16. L. Wu, Z. M. Li and Q. Ye, *J. Ind. Microbiol. Biotechnol.*, **36**, 1289 (2009).
17. Y. C. Park, H. J. Lee, C. S. Kim and J. H. Seo, *J. Microbiol. Biotechnol.*, **23**(4), 560 (2013).
18. J. Cheng, W. Zhuang, N. N. Li, C. L. Tang and H. J. Ying, *Lett. Appl. Microbiol.*, **64**, 73 (2017).
19. Y. C. Park, J. H. Choi, G. N. Bennett and J. H. Seo, *J. Biothchnol.*, **121**, 508 (2006).
20. E. Belda, A. Sekowska, F. F. Le, A. Morgat, D. Mornico, C. Ouzounis, D. Vallenet, M. Claudine and A. Danchin, *Microbiology*, **159**, 757 (2013).
21. X. Y. Zhao, J. J. Liu, J. X. Zhang and P. W. Li, *Food Drug.*, **7**(3), 23 (2005).
22. N. A. Shevchuk, A. V. Bryksin, Y. A. Nusinovich, F. C. Cabello, M.

Sutherland and S. Ladisch, *Nucleic Acids Res.*, **32**(2), e19 (2004).
23. X. Yan, H. J. Yu, Q. Hong and S. P. Li, *Appl. Environ. Microb.*, **74**(17), 5556 (2008).
24. J. Spizizen, *Proc. Natl. Acad Sci. U.S.A.*, **44**, 1072 (1958).
25. Y. J. Tian, Y. X. Fan, J. J. Liu, X. Y. Zhao and W. Chen, *Electron. J. Biotechnol.*, **19**, 41 (2016).
26. J. Saxena and R. S. Tanner, *World J. Microbiol. Biotechnol.*, **28**(4), 1553 (2012).
27. P. D. Wulf, W. Soetaert, D. Schwengers and E. J. Vandamme, *J. Ind. Microbiol.*, **17**(2), 104 (1996).